Enrichment of Adipose-derived Mesenchymal Stem Cells Using Resveratrol

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by

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1. Summary

Mesenchymal stem cells (MSCs) hold great promise for the application of tissue engineering to orthopedic problems such as critical-size defects. Cell sorting has indicated a benefit to enriching the MSC population, but is undesired for clinical applications. Pharmacological enrichment utilizing adipogenic inhibitors such as resveratrol represent a clinically viable alternative. Cells were isolated from rats, and treated with differentiation media to verify multipotency, and were then treated with 0, 12.5, and 25 μM resveratrol in growth media and osteogenic media. MSC and osteoprogenitor (OPC) populations were measured using flow cytometry and OPC quality was assessed with osteocalcin production, osteoprotegerin production, and alkaline phosphatase activity. Resveratrol increased the population percent and cell number of MSCs in both growth and osteogenic media, but only increased the number of OPCs in growth media. In both media types resveratrol increased alkaline phosphatase activity and osteocalcin levels. Resveratrol enriches ADMSCs for mesenchymal stem cells and osteoprogenitors.

2. Introduction

The repair of large bone defects is one of the major problems currently facing orthopedics. The two current treatments are autografts and allografts [1]. Both treatment options have numerous problems and complications. Autografts suffer from a limited supply of tissue and insufficient structural integrity. Allografts have the advantage of providing structural integrity, but only in the short-term, and have a high failure rate. The lack of an effective treatment option is due largely to the inability of the graft to effectively integrate with the patient's existing bone and to revascularize [2]. Stem cell therapy has the potential to provide a solution in healing large bone defects.

Stem cells are a type of cell with the capabilities of self-renewal and multipotency. An important group of stem cells is mesenchymal stem cells (MSCs). These cells have the ability to differentiate into many tissue pathways such as osteogenic, chondrogenic, and adipogenic. Their multipotency and ability to avoid allogeneic rejection make them a prime candidate for tissue engineering [3]. The traditional source of MSCs is bone marrow, which are typically defined as (a) able to adhere to plastic; (b) exhibiting the following surface marker phenotype: $CD14^-$, $CD19^-$, $CD34^-$, $CD45^-$, HLA-DR^-, $CD73^+$, $CD90^+$, $CD105^+$; and (c) capable of multipotent differentiation [4]. More recently CD271 has been detected on bone marrow-derived MSCs, and has been proposed as a marker for their isolation [5, 6]. Their clinical isolation has been accompanied with a number of adverse events such as morbidity, pain, and low yield. These complications, particularly the low yield, highlight the need for research into the characterization of new sources for MSCs.

Adipose tissue has been shown to contain a supply of multipotent progenitor cells, called adipose-derived mesenchymal stem cells (ADMSCs), capable of maturing along the connective tissue lineages [7-9]. It has been shown to yield more stem cells per tissue amount than bone marrow [10]. The phenotype of ADMSCs has been described in the literature, indicating many similarities with bone marrow-derived MSCs as described above; particularly $CD34^-$, $CD45^-$, $CD73^+$, $CD90^+$, and $CD271^+$ [11-18].

Along with mesenchymal stem cells, adipose tissue also yields a variety of other cells such as preadipocytes, osteoprogenitor cells, vascular smooth muscle cells, and vascular endothelial cells [10]. Thus far no clear phenotype of osteoblast and osteoprogenitor surface markers has been developed. Osteocytes, but not osteoblasts have been shown to selectively express E11, making it a potential late-stage osteogenic marker [19]. Osteocalcin (OCN), although not typically thought of as a surface marker, has been used previously as such to detect circulating osteoblasts from the bone marrow [20]. Finally α-smooth muscle actin has been shown to identify circulating osteoprogenitor cells following osteoblast ablation [21].

In order to effectively utilize ADMSCs clinically, the undesired cells need to be separated from the mesenchymal stem cells and desired progenitors [22, 23]. A number of groups are currently looking at a variety of ways to increase the overall multipotency of this heterogeneous population, or to enrich the stem cells. The current technique is to use fluorescent activated cell sorting (FACS) to sort the cells based on the presence of certain surface markers associated with mesenchymal stem cells [22, 24-26].

The use of FACS to enrich MSCs poses a couple of problems to clinical use. First, FACS requires the use of animal derived antibodies. These antibodies are expensive and pose a concern for clinical use in that they could elicit a adverse reaction in the patient. Secondly, when using the FACS technique sterility can be difficult to maintain, and infected cell grafts could ruin a tissue engineered implant.

The differentiation of these cells is determined by many factors including local environment and chemical signals, and hormones [27, 28]. A reciprocal relationship exists between osteogenesis and adipogenesis; thus inhibition of one leads to activation of the other. It has recently been shown that resveratrol and isonicotinamide enhance osteogenesis, and nicotinamide enhances adipogenesis through inhibition and activation of Sirt1 [29].

A pharmacological approach could solve all of the problems posed by FACS enrichment. Specifically, the use of resveratrol as an adipogenic inhibitor could provide an inexpensive and effective pharmacological means to enrich mesenchymal stem cells, and to select for the desired progenitors. Successful pharmacological enrichment of ADMSCs for osteoprogenitors could potentially provide a novel treatment for critical sized bone defects and regenerative medicine.

The project aims to accomplish: (i) the verification of a protocol to isolate mesenchymal stem cells from adipose tissue, (ii) the establishment of an adipose-derived stem cell model for further study in bone regeneration and engineering, and (iii) the investigation of resveratrol as a pharmacological enriching agent for adipose-derived mesenchymal stem cells and osteoprogenitors.

In order to verify an isolation protocol for MSCs from adipose tissue, we differentiated the cells with adipogenic media (AM), chondrogenic media (CM), and osteogenic media (OM) and assessed the presence of differentiation markers for each treatment. To establish an ADMSC model, we defined two populations of interest: mesenchymal stem cells (MSCs) and osteoprogenitor cells (OPCs). MSCs were defined as CD 271^{+}, CD 73^{+}, and CD 45^{-}. OPCs were defined as $E11^{+}$ and OCN^{+}. The potential of resveratrol as an enriching agent was assessed by treating isolated rat ADMSCs with 0, 12.5, and 25 μM resveratrol, and measuring the population changes of MSCs, OPCs, and the increase of the osteogenic markers: osteocalcin, osteoprotegerin, and alkaline phosphatase activity.

3. Results

Verification of Multipotency

Adipose tissue has been shown to yield a population of multipotent mesenchymal stem cells. In order to verify the multipotency of the isolated cell population, rat ADMSCs were differentiated into osteoblasts (Figure 1), adipocytes (Figure 2), and chondrocytes (Figure 3). After 14 days, cells treated with osteogenic media (OM) showed phosphate deposition (Figure 1a). Osteogenic media significantly increased alkaline phosphatase activity over growth media (GM) throughout a 28 day induction. Additionally, the cells grown in GM showed little increase in activity. Activity peaked at the release over 40 nMol/mg Prot./min of phosphate in the osteogenic group (Figure 1b). Throughout differentiation, ADMSCs expressed Runx2, alkaline phosphatase, and osteopontin (Figure 1c).

After 14 days of treatment in adipogenic media (AM), ADMSC stained positive for lipid accumulation (Figure 2a). Adipogenic media increased adiponectin levels throughout a 28 day treatment, over cells grown in GM (Figure 2b), which showed no significant change in adiponectin levels. Adiponectin levels peaked at just under 100 pg/10^5 cells. ADMSCs expressed leptin, peaking after 14 days, and PPARγ2 during adipogenic differentiation (Figure 2c).

Adipose-derived mesenchymal stem cells treated with chondrogenic media (CM) stained positive for proteoglycan production (Figure 3a). ADMSCs grown in chondrogenic media expressed collagen I, collagen II, aggrecan, and collagen X. Collagen I expression was present throughout the entire 21 day differentiation of the

cells. Expression of collagen II and aggrecan began after 14 days in culture, where as collagen X expression appeared after 21 days (Figure 3b).

Dose Determination and Effect on Osteogenic Markers

In order to determine the proper concentration range, ADMSCs were treated with 0, 12.5, 25, and 50μM resveratrol in GM and OM. In GM, cell number varied significantly in each treatment with respect to the control (Figure 4a). The lowest treatment increased cell number from 4.5 x 10^5 to 5.0 x 10^5 cells. Treatments of 25 and 50μM resveratrol reduced cell number to 4.1 an 0.2 x 10^5 cells respectively. In OM, cell number again varied little across the concentrations. Resveratrol increased alkaline phosphatase activity in a dose-dependent manner in both media types through 25μM, peaking at 9 nMol Pi/mg Prot./min for GM and 23 nMol Pi/mg Prot./min in OM. The 50μM treatment increased activity significantly in GM and OM to 2 and 6 nMol Pi/mg Prot./min respectively (Figure 4b).

Adipose-derived mesenchymal stem cells treated with resveratrol increased levels and activity of certain osteogenic markers such as osteoprotegerin, osteocalcin, and alkaline phosphatase activity. Resveratrol increased osteoprotegerin levels in GM dose-dependently, but showed no change in OM (Figure 5a). Resveratrol slightly increased osteocalcin levels over control in both media types (Figure 5b). The treatment increased alkaline phosphatase activity in GM and OM in a dose-dependent manner (Figure 5c and 5d).

Resveratrol treatment improved osteogenic cell morphology (Figure 6). Growth media vehicle-control cells (Figure 6a) exhibited a fibroblastic morphology typical of

mesenchymal stem cells, excepting for the lipid vacuoles present in many of the cells. In osteogenic media, vehicle control cells (Figure 6b) exhibited a spread morphology with a few cells developing lipid vacuoles. Cells treated with 25μM resveratrol in GM (Figure 6c) had a morphology in between both vehicle controls. Most cells had a fibroblastic shape, but many were more spread, resembling the OM vehicle control. Cells treated with 25μM resveratrol in OM (Figure 6d) had a more compacted morphology and appeared to be developing nodules. In both GM and OM 25μM resveratrol (Figures 6c and 6d respectively) reduced lipid accumulation relative to the vehicle controls (Figure 6a and 6b respectively).

Resveratrol Enriches ADMSCs for MSCs and OPCs

Resveratrol treatment enriched the MSC and osteoprogenitor (OPC) populations in cultured ADMSC. Treatment with resveratrol increased the number of MSCs in both GM and OM, with the 25μM groups showing a significant increase over the 12.5μM groups. In GM resveratrol treatment increased the cell number by 5-fold, and in OM by 2-fold (Figure 7a). Population percentage showed a similar trend as the cell numbers, except that the two media types showed little difference, with 4-fold and 2-fold increases in GM and OM respectively (Figure 7b).

In addition to enriching the MSC population, resveratrol treatment enriched the population of OPCs. The number of OPCs increased significantly with resveratrol treatment in GM, with a 3-fold increase at the highest dose. The treatment had no effect on the number of OPCs in OM. However, OM significantly increased the number and population percent of OPCs over that of GM in nearly every group (Figure 8a). Similar

results were seen in terms of population percentage of OPCs. Treatment increased the percent of OPCs in GM, with a 2-fold increase at the highest dose. Resveratrol had no effect on the percent of OPCs in OM, however (Figure 8b).

Effect of Resveratrol on Human ADMSCs

In order to examine the effect of resveratrol on human ADMSCs and determine an appropriate concentration range, cells were isolated from donated human adipose tissue, and treated with 0, 12.5, 25, 50, and 100 μM resveratrol. Resveratrol increased alkaline phosphatase activity in GM at 12.5μM. Higher concentrations decreased activity below the control. In OM, resveratrol increased alkaline phosphatase activity dose-dependently to a maximum at 50μM. The highest dose showed activity similar to control (Figure 9a). In both GM and OM, resveratrol treated cells showed a reduced cell number, and altered cell morphology (Figure 9d and 9e). Human ADMSCs grown in 0μM + GM showed a good MSC morphology, and were near confluent after 7 days (Figure 9b). Those treated with 100μM resveratrol however, exhibited an elongated morphology with many dendritic appendages stretching across the plate. These cells not proliferate as well, and there were markedly fewer cells than the vehicle control (Figure 9d). Treatment with resveratrol-free osteogenic media exhibited an osteoblastic phenotype (Figure 9c). These cells too proliferated well and were confluent after 7 days. Similar to their GM counterpart, the cells treated with 100μM resveratrol showed a rather dendritic phenotype, and had a reduced cell number (Figure 9e).

4. Discussion

Mesenchymal stem cells hold great promise for the application of tissue engineering to orthopedic problems such as critical-size defects. Unfortunately significant problems must be solved before there can be widespread application of stem cell therapies, such as timely, safe, and cost-effective means of ensuring the desired cellular differentiation and tissue regeneration. Enrichment through cell sorting has posed a promising technique to ensure the proper differentiation of MSCs, but carries with it a host of problems including expense, potential for adverse reactions, and difficulty in maintaining sterility. With regards to orthopedic applications, adipogenic inhibitors, such as resveratrol, present a unique opportunity for the development of a pharmacological enrichment for both medical therapies and further inquiry.

Resveratrol treatment has been shown to provide a potent enrichment for MSCs and OPCs. It increases the number and percentage of MSCs in both media types, thereby enriching the MSC population. Resveratrol increased the number and percentage of OPCs in GM only, and had no effect in OM. Even though resveratrol had no effect on the OPC population in OM, there were generally more OPCs in each OM group than GM group. The differences between media types are likely due to the effect of the differentiation media. The dexamethasone in the OM is likely pushing the cells to become osteoblasts so strongly that it overshadows the resveratrol effect. These results suggest that an improved enrichment would begin with resveratrol in GM to elevate the number of MSCs, followed by treatment with OM to push them to differentiate.

Although osteocalcin levels did not vary significantly, osteoprotegerin did increase slightly in GM. Since alkaline phosphatase activity increased in both media types and the treatment lasted 7 days, the cells are likely in the early stages of osteogenesis, and may not have begun significant production of osteocalcin and osteoprotegerin.

The isolation method of ADMSCs has been tailored to isolate only adherent stromal cells, and to avoid any fibroblast contamination. The alkaline phosphatase results initially indicate a different optimum concentration for each media. The cell culture images suggest a different story. In this particular case, the donor tissue was mammarian, which did not initially pose any cause for concern. During the treatment, most notably at the higher concentrations, the cells lost the fibroblastic morphology typical to MSCs, and took on a more elongated morphology with numerous dendritic appendages. Moreover, the cells treated with 100μM resveratrol appeared to have more in common than their 0μM counterparts in that they had the same morphology and showed similar growth. Further examination suggested that these may be mammarian epithelial cells. These cells are found in the stroma of the tissue, and may be an unavoidable contaminant from this tissue.

5. Conclusion

Enrichment of mesenchymal stem cells has been shown to increase their effectiveness in tissue repair and regeneration. Resveratrol enriches adipose-derived mesenchymal stem cells for both MSCs and OPCs by increasing the overall number and percentage of total cells. Current theories indicate that different donor sites may yield differing qualities of cells. Our results with human ADMSCs indicate that this is very likely the case and that certain tissues may present undesirable and unavoidable contaminants, making them undesirable for certain applications.

6. Experimental Procedures

6.1 ADMSC Differentiation Verification

Cell Isolation and Culture: Inguinal fat pads, subcutaneous fat surrounding the thigh, were harvested from 100 - 125g sprague dawley rats according to approved IACUC protocol. The tissue was washed three times in Hank's Balanced Saline Solution (HBSS), and digested in trypsin for 30 minutes at 37 ˚C. The tissue was then cut into smaller pieces and digested in collagenase IA (9125 units) and dispase (75 units) until completely digested. The upper layer of adipocytes was removed, and the cell suspension was filtered through a 40μm cell strainer. The digestion was quenched with GM (Lonza, Basel, Switzerland) and the cells were pelleted (2000 *g* for 10 minutes). The cells were plated at 5,000 cells/cm^2 and washed with phosphate buffered saline (PBS) after 24 and 48 hours. Cells were grown in 24 or 6 well plates in growth media (GM), adipogenic media (AM), osteogenic media (OM) and chondrogenic media (CM) for 7, 14, 21, and 28 hours.

Adipocyte Staining with Oil Red O: Samples were washed twice with PBS, and fixed with 10% formalin. They were then stained with a 0.3% Oil Red O/0.4% dextrin solution for 20 minutes. The cells were rinsed in running water, counterstained with Gill II hematoxylin, and rinsed with distilled water. They were blued in Scott's solution, and imaged via light microscopy.

Adiponectin: Twenty-four hours prior to harvest, cell culture media were replace with fresh media. At harvest, cell culture media were removed and stored at -20˚C. Cell monolayers were trypsinized for 10 minutes to remove the cells. The reaction was

quenched with full medium, and the cells were centrifuged (2000 *g* for 10 minutes) and resuspended in PBS. Cells were counted, resuspended in 0.05% Triton-X 100, and stored at -20˚C. After the harvest of all groups, cell media were thawed, and adiponectin levels were measured via adiponectin ELISA (R&D Systems Minneapolis, MN).

Von Kossa Phosphate Deposition Staining: Samples were washed twice with PBS, and fixed with 10% formalin. On the day of the staining, they were rehydrated to distilled water and stained in 5% silver nitrate for 20 minutes under UV light. The samples were rinsed in distilled water, and stained in 5% sodium thiosulfate for 3 minutes. They were washed with distilled water and counterstained for 5 minutes with nuclear-fast red. Samples were washed again in water and imaged via light microscopy.

Alkaline Phosphatase Activity: Cell monolayers were lysed with 0.05% Triton-X 100 and stored at -20˚C until assay date. Alkaline phosphatase activity was measured as the release *p*-nitrophenol from *p*-nitrophenolphosphate. Activity was normalized to protein concentration and reaction duration.

Proteoglycan Staining with Toluidine Blue: Samples were washed twice with PBS and fixed in 10% formalin. On the day of staining, they were rehydrated to distilled water and stained in 0.1% toluidine blue solution for 5 minutes. The samples were washed in distilled water and imaged via light microscopy.

Gene Expression: ADMSCs were grown in 6 well plates treated with AM, OM, GM, and CM for 7, 14, and 21 days. On the day of the harvest, cell media were aspirated, and RNA was isolated with a trizol-chloroform extraction. Samples were then converted into cDNA using random primers. Samples were then analyzed via PCR for the presence of runx2, alkaline phosphatase, osteopontin, leptin, PParγ2, collagen I, collagen II, aggrecan, collagen X, and GapDH.

6.2 Physiological Response of rat MSCs

Cell Isolation and Culture: Cells were isolated as described above. The cells were grown in T-75 flasks and 24 well plates for 7 days in GM and OM supplemented with 0, 12.5, or 25μM resveratrol.

Dose Determination: ADMSCs were grown in 24 well plates in GM and OM supplemented with 0, 12.5, 20, 50μM resveratrol for 7 days. On the day of the harvest, cell monolayers were trypsinized for 10 minutes to remove the cells. The reaction was quenched with full medium, and the cells were centrifuged (2000 *g* for 10 minutes) and resuspended in PBS. Cells were counted, resuspended in 0.05% Triton-X 100, and stored at -20˚C. Alkaline phosphatase activity was measured as described above.

Cell Morphology: Every 24 hours, an image of each group was captured via light microscopy.

Flow Cytometry: Cells were washed with PBS, detached with 0.25% trypsin, and pelleted by centrifugation (2000 *g* for 10 minutes). The cells were counted, and 500,000 cells were aliquoted for flow cytometry. The cells were washed with the wash buffer

from the BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD Franklin Lakes, NJ). The cells for MSC measurement were incubated with antibodies for CD 271 (Santa Cruz Biotechnology Santa Cruz, CA), CD 73 (BD Franklin Lakes, NJ), CD 45 (BD Franklin Lakes, NJ), and an Allophycocyanin conjugated Goat anti-Mouse secondary antibody (R&D Systems Minneapolis, MN). The cells for OPC measurement were incubated with antibodies for osteocalcin (Santa Cruz Biotechnology Santa Cruz, CA), E11 (Sigma St. Louis, MO), a FITC conjugated anti-Rabbit secondary antibody (ABCam Cambridge, MA), and an Allophycocyanin conjugated Donkey anti-Goat secondary antibody (R&D Systems Minneapolis, MN) in 1% bovine serum albumin in PBS for 20 minutes. In between incubations the cells were washed twice. Populations were then measured on a BD LSR Flow Cytometer relative to isotype control.

Osteogenic Differentiation Markers: Twenty four hours prior to harvest, cell medium was replaced with fresh medium. This medium was collected, and the cells were lysed with 0.05% Triton-X 100. Alkaline phosphatase activity was measured as the release *p*-nitrophenol from *p*-nitrophenolphosphate. Osteocalcin levels in the conditioned media were measured via the Human Osteocalcin Radioimmunoassay kit (Biomedical Technologies Stoughton, MA) as reported previously. Osteoprotegerin levels in the conditioned media were measured via the Osteoprotegerin ELISA (R&D Systems Minneapolis, MN).

6.3 Effect of Resveratrol on Human ADMSCs

Cell Isolation and Culture: Human adipose tissue was donated during a breast reduction following an approved IRB protocol. After receipt of the tissue, ADMSCs were isolated as described above.

6.4 Statistical Analysis

Data were analyzed with a one-way ANOVA to determine the presence of significant differences between groups. If the ANOVA yielded alpha ≤ 0.05, a t-test was used with a bonferroni's modification was used to determine significance. Adiponectin levels, surface characterization, total cell number, and alkaline phosphatase activity with resveratrol represent a mean of three ± standard error. All symbols represent $\alpha \leq 0.05$ (# with respect to same timepoint, • relative to next lowest treatment, and * with respect to vehicle control). Induction alkaline phosphatase activity, osteoprotegerin levels, and osteocalcin levels represent a mean of 6 ± standard error.

7. Acknowledgements

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9. Figures

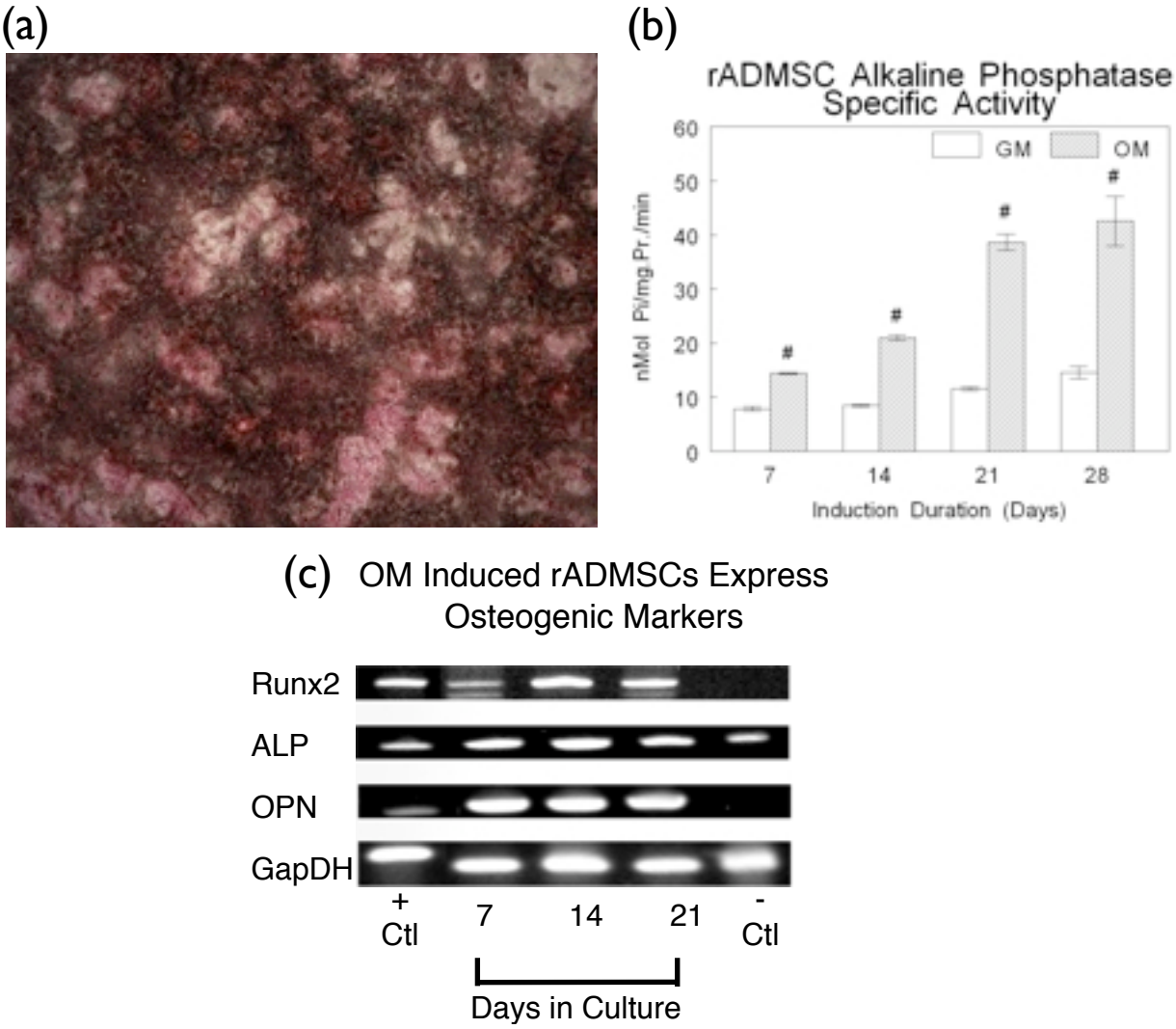

Figure 1: Adipose-derived Mesenchymal Stem Cells Differentiate into Osteoblasts
ADMSCs were grown for 14 days in OM, and showed positive for phosphate deposition via Von Kossa staining (a). ADMSCs showed increased alkaline phosphatase activity over 28 days of induction in OM. Cells grown in GM showed little change in alkaline phosphatase activity (b). ADMSCs treated with OM expressed Runx2, ALP (alkaline phosphatase), and OPN (Osteopontin) throughout a 21 day induction (c). Alkaline phosphatase activity data represent mean ± standard error (n = 6). Data were analyzed with a one-way ANOVA followed by a two-tailed t-test with a bonferroni's modification (# represents $\alpha \leq 0.05$ with respect to same time point control).

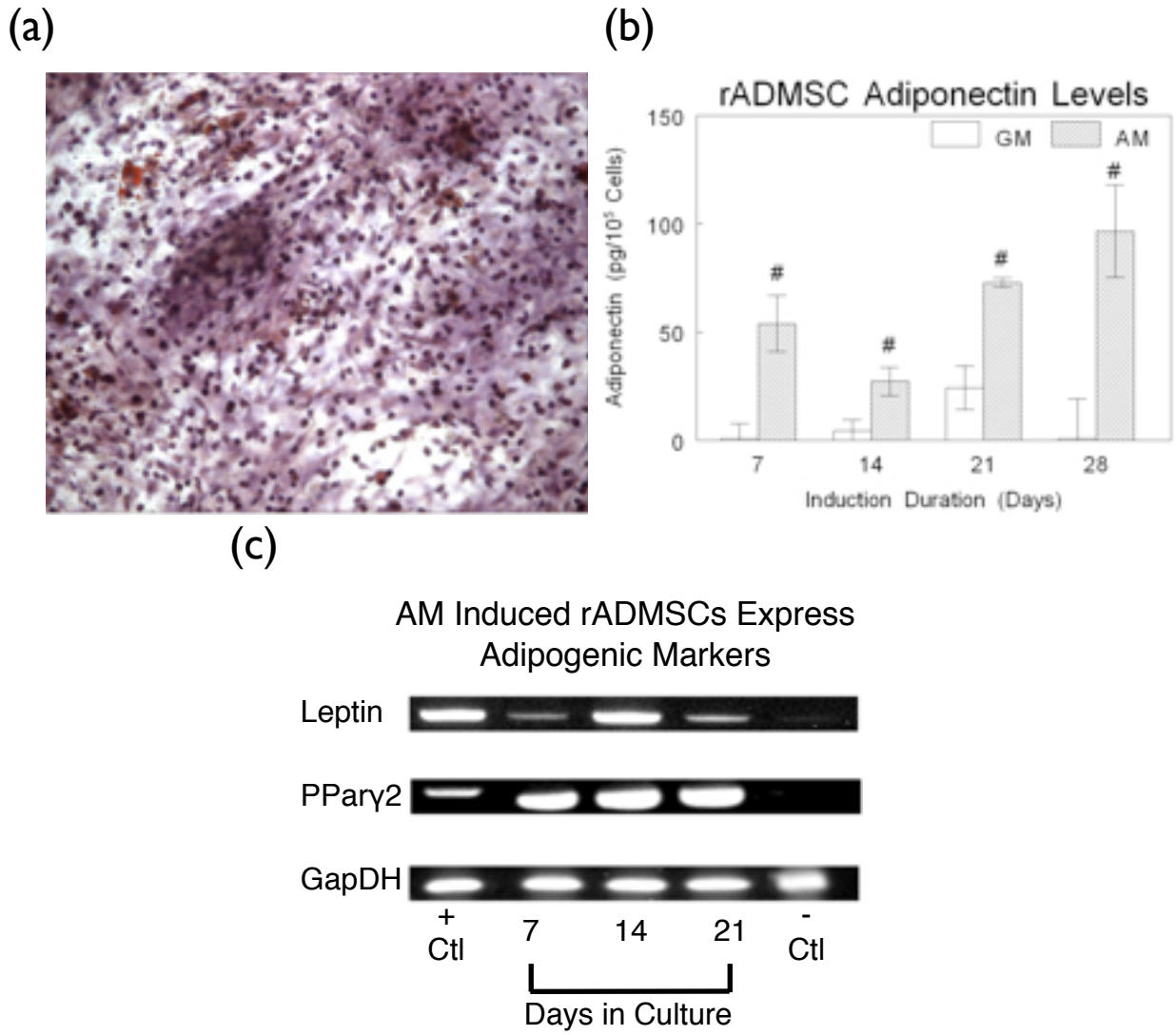

Figure 2: Adipose-derived Mesenchymal Stem Cells Differentiate into Adipocytes
ADMSCs were grown for 14 days in AM, and showed positive for lipid accumulation via Oil Red O staining (a). ADMSCs showed increased adiponectin levels over 28 days of induction in AM. Cells grown in GM showed little change in adiponectin levels (b). ADMSCs treated with AM expressed Leptin, and PPARγ2 throughout a 21 day induction (c). Adiponectin data represent mean ± standard error (n = 3). Data were analyzed with one-way ANOVA followed by a two-tailed t-test with a bonferroni's modification (# represents $\alpha \leq 0.05$ with respect to same time point control).

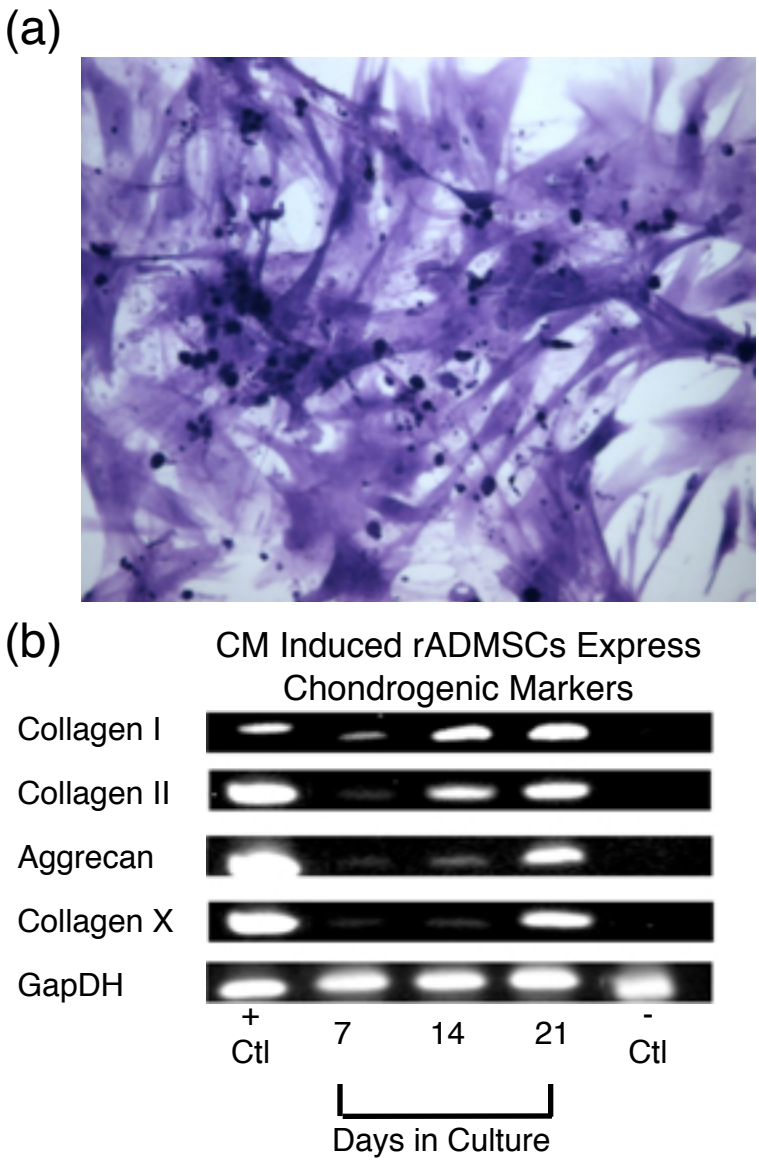

Figure 3: Adipose-derived Mesenchymal Stem Cells Differentiate into Chondrocytes
ADMSCs were grown for 14 days in CM, and showed positive for proteoglycan production via toluidine blue staining (a). ADMSCs treated with CM expressed Collagen I, Collagen II, Aggrecan, and Collagen X throughout a 21 day induction (b).

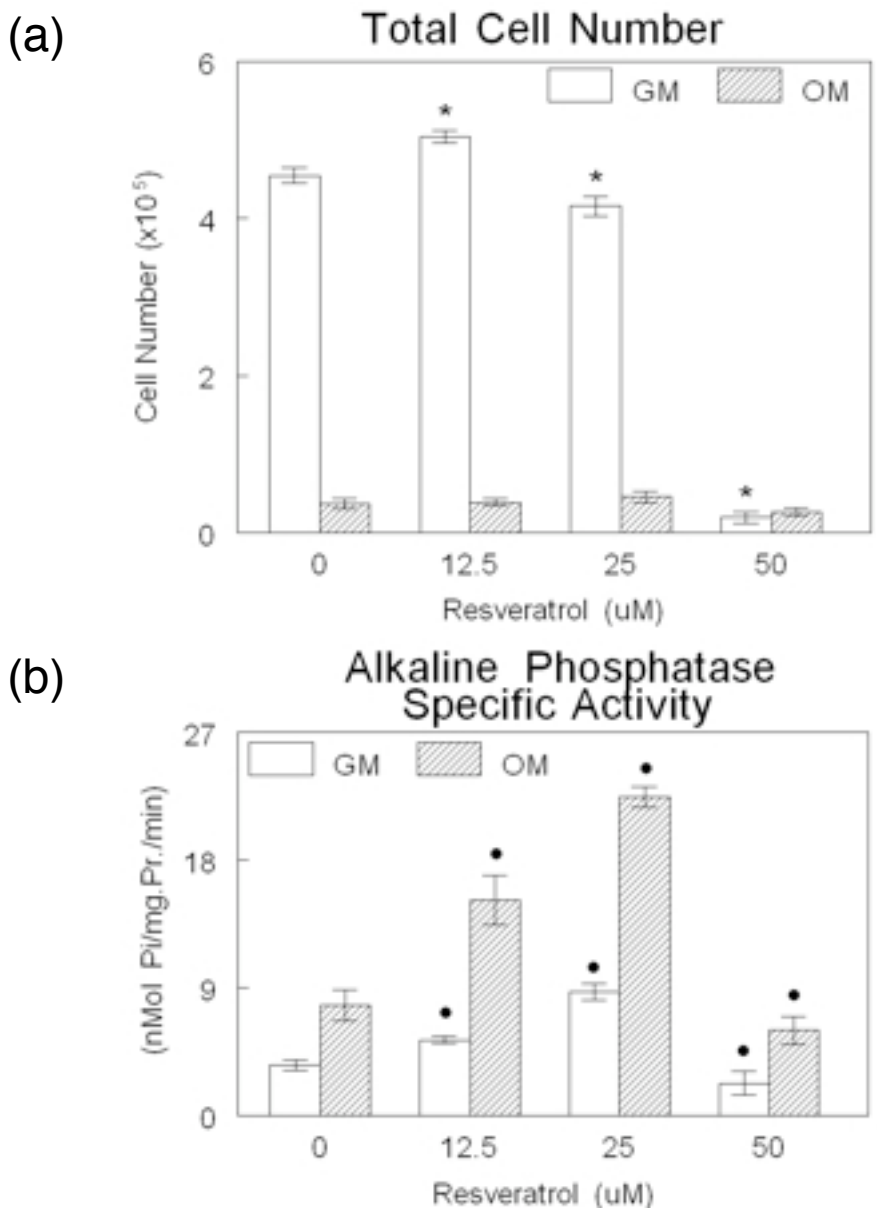

Figure 4: Determination of Dose

Resveratrol had no effect on cell number in either GM or OM up to 50 μM (a). At 50 uM, resveratrol reduced cell number significantly. Alkaline phosphatase activity increased dose-dependently up to 50 μM, at which point it decreased below baseline (b). Values represent mean ± sem (n = 3). Data were analyzed with a one-way ANOVA follwed by a two-tailed t-test with a bonferroni's modification (• represents $\alpha \leq 0.05$ with respect to same time point control and * represents $\alpha \leq 0.05$ with respect to vehicle control).

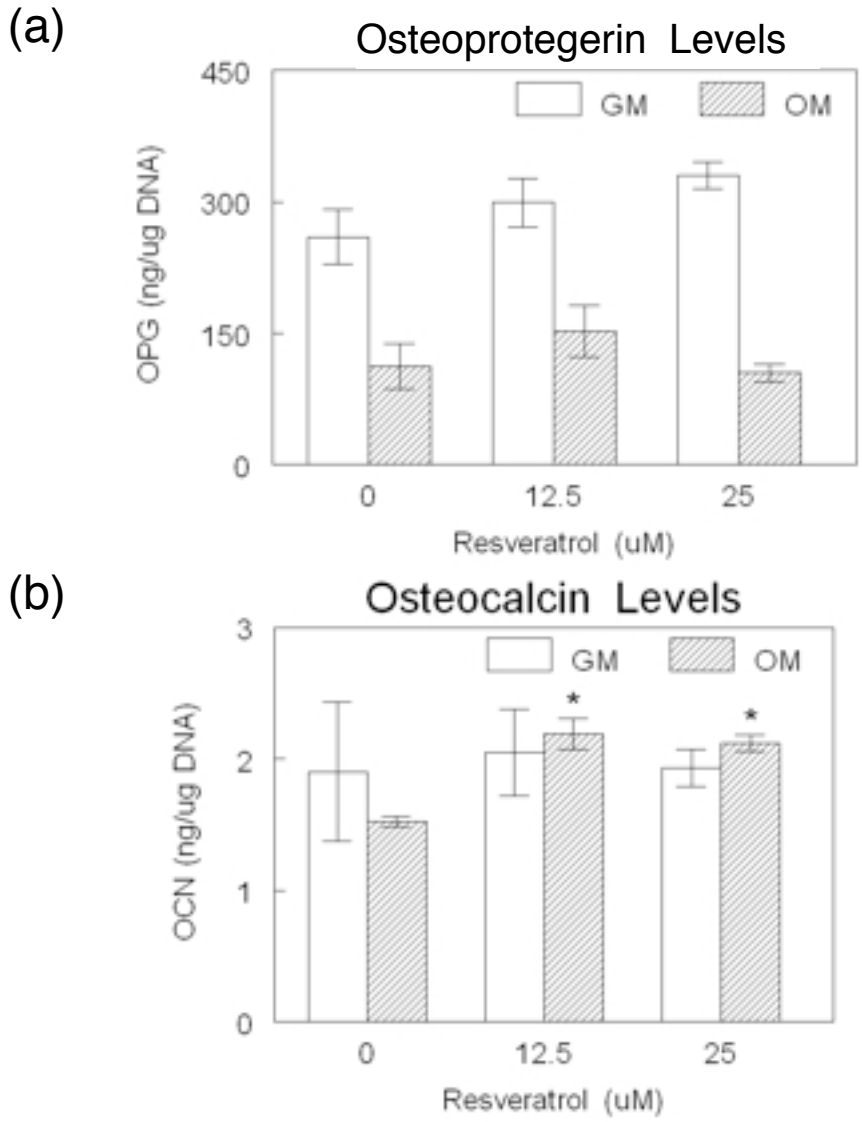

Figure 5: Resveratrol increases Osteogenic Markers

Resveratrol increased levels of secreted osteoprotegerin in a GM. In OM resveratrol had no effect on osteoprotegerin levels and for all treatments values were lower than their corresponding GM group (a). Treatment with resveratrol increased osteocalcin levels over vehicle-control in both GM and OM. There was no difference in osteocalcin levels between the two media conditions (b). Treatment increased alkaline phosphatase activity in GM and OM (c). Values represent mean ± sem (n = 6). Data were analyzed with a one-way ANOVA followed by a two-tailed t-test with a bonferroni's modification (* represents $\alpha \leq 0.05$ with respect to vehicle control).

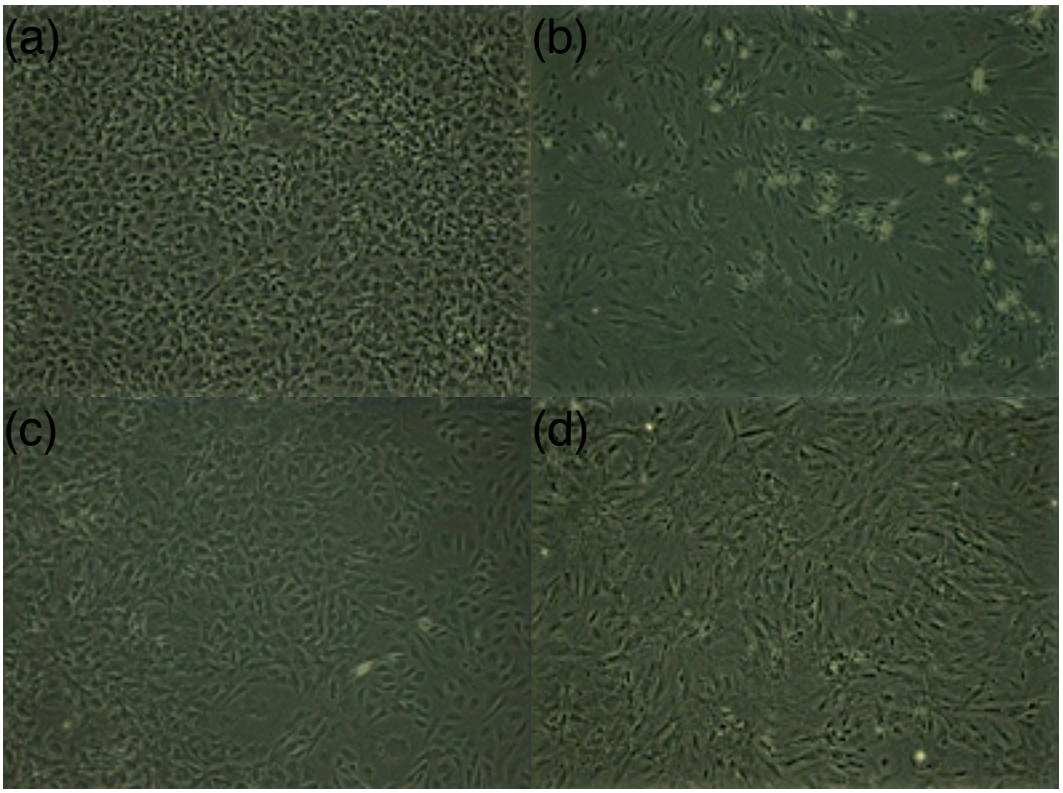

Figure 6: Resveratrol Enhances Osteogenic Morphology
ADMSCs grown in GM (a, c) and OM (b, d) exhibit a markedly different morphology. ADMSCs in GM with no resveratrol had fibroblastic morphology (a), whereas those in OM without resveratrol (b) had a more spread morphology. Cells treated with 25μM resveratrol in GM seemed to show a mix between the fibroblastic and spread morphology (c). ADMSCs treated with OM and 25μM resveratrol show a more compacted morphology, and appeared to be developing nodes. Additionally, both GM vehicle control cells (a) and OM vehicle control cells (b) showed the development of lipid vacuoles in many cells, whereas those treated with 25μM resveratrol showed reduced lipid accumulation (c, d).

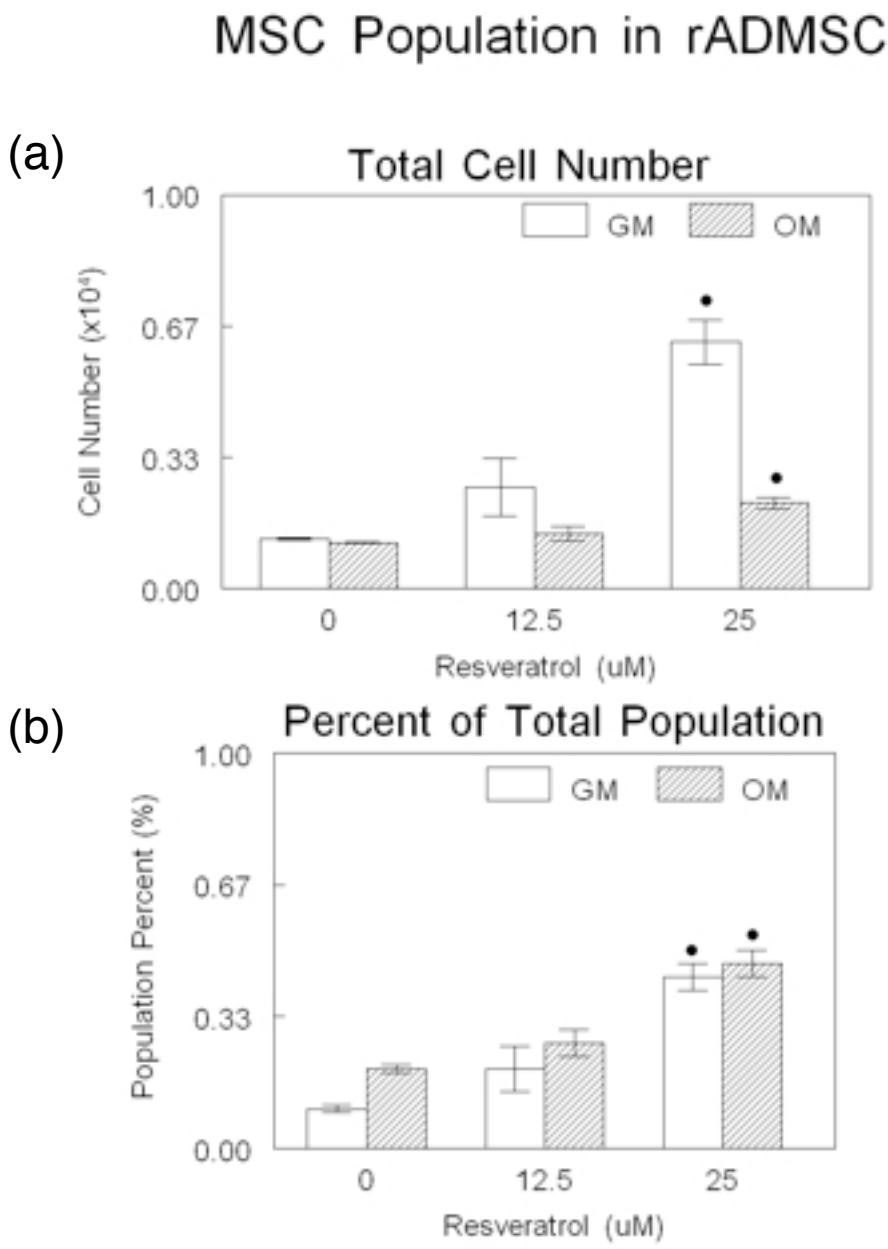

Figure 7: Resveratrol Enriches the MSC Population in ADMSCs

MSCs were defined as CD 271+, CD73+, and CD 45-. Resveratrol treatment increased the number of MSCs in a dose-dependent manner in both GM and OM. In both treatments, the number of MSCs was greater in GM than in OM (a). Resveratrol increased the MSC population percentage in a dose-dependent manner in both media types. In terms of percent of total cell population, there was little difference between the media conditions in both treatments (b). Values represent mean ± sem (n = 3). Data were analyzed with a one-way ANOVA follwed by a two-tailed t-test with a bonferroni's modification (● represents $\alpha \leq 0.05$ with respect to 12.5μM group).

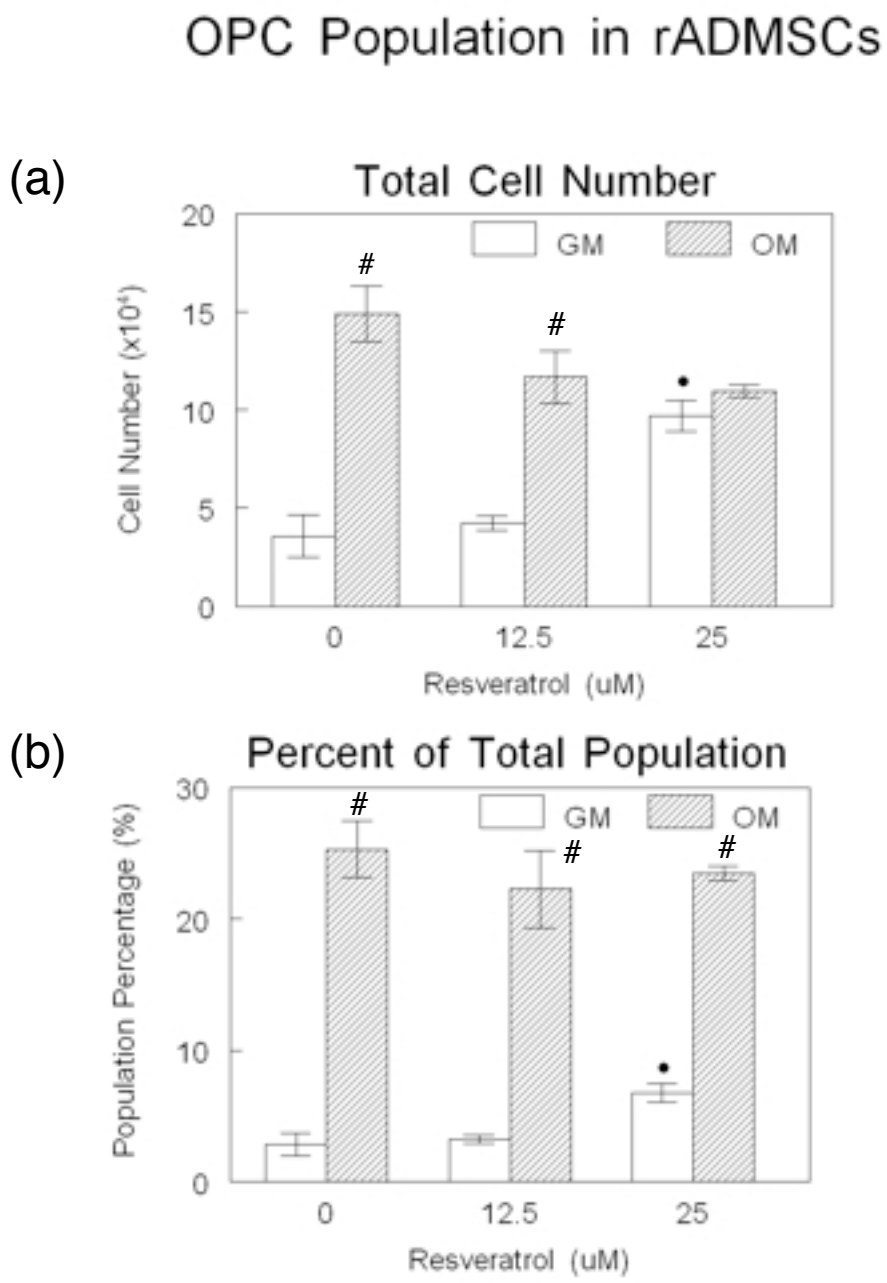

Figure 8: Resveratrol Enriches the OPC Population in ADMSCs

OPCs were defined as OCN+ and E11+. Resveratrol treatment increased the number of OPCs in GM. It had no effect on the number of OPCs in OM. In all treatments, the number of OPCs was greater in OM than in GM (a). Resveratrol increased the OPC population percentage in GM. It had no effect on the population percent in OM, which was significantly greater than all GM samples (b). Values represent mean ± sem (n = 3). Data were analyzed with a one-way ANOVA followed by a two-tailed t-test with a bonferroni's modification (• represents $\alpha \leq 0.05$ with respect to 12.5μM group and # represents $\alpha \leq 0.05$ with respect to same concentration GM group).

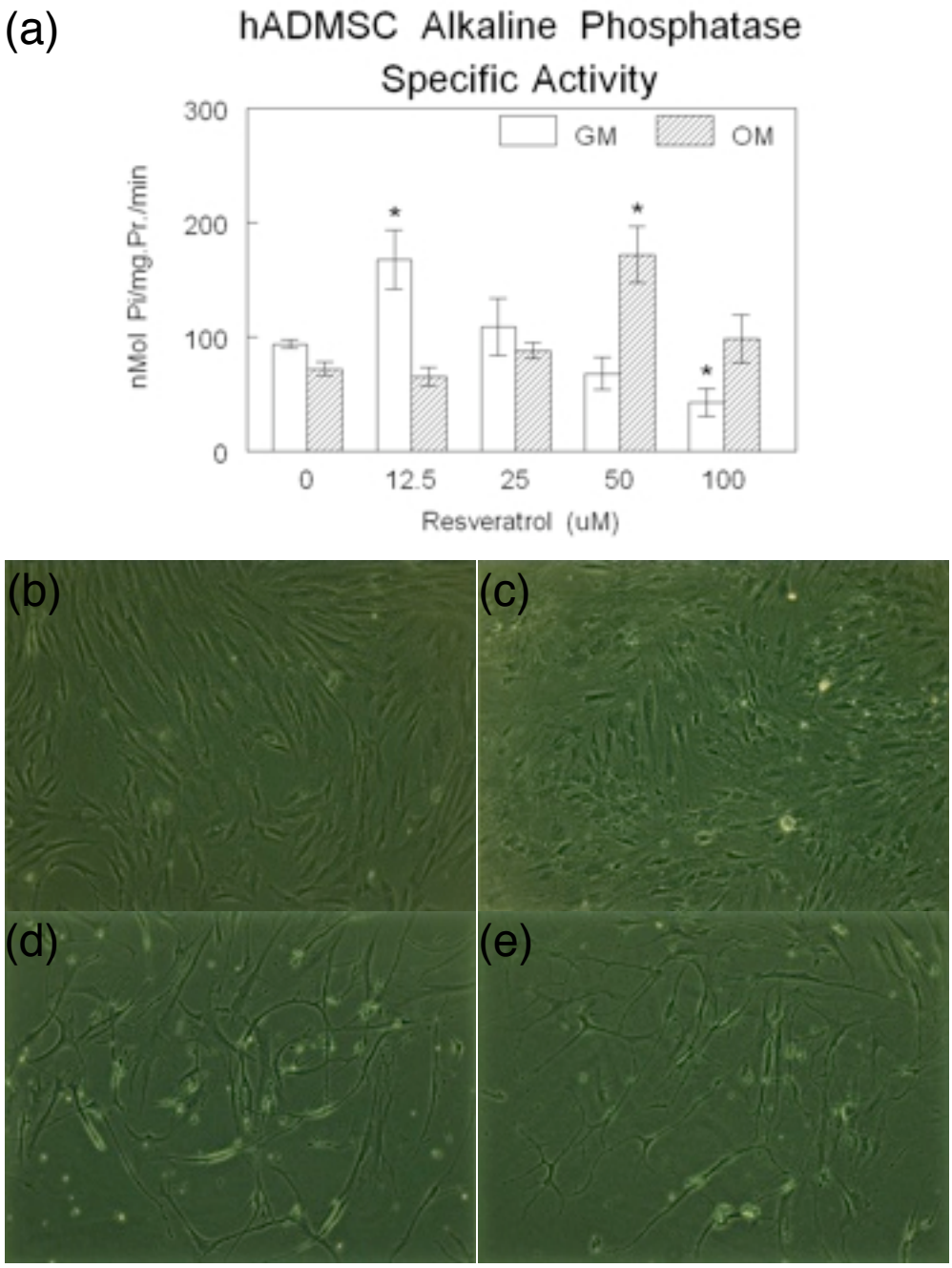

Figure 9: Effect of Resveratrol on Human ADMSCs

Resveratrol increased activity of alkaline phosphatase in both GM and OM, but peaked at different doses. In GM 12.5μM induced the highest activity, while in OM 50uM caused the highest activity (a). Resveratrol reduced the number of cells in both GM (b, d) and OM (c, e). Cells treated with 100μM resveratrol (d, e) exhibited a more elongated and dendritic morphology than vehicle control groups (b, c). Values represent mean ± sem (n = 6). Data were analyzed with a one-way ANOVA follwed by a two-tailed t-test with a bonferroni's modification (* represents $\alpha \leq 0.05$ with respect to same time point control).